

# Confirmation of diosmetin 3-*O*-glucuronide as major metabolite of diosmin in humans, using micro-liquid-chromatography–mass spectrometry and ion mobility mass spectrometry

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**Abstract** Diosmin is a flavonoid often administered in the treatment of chronic venous insufficiency, hemorrhoids, and related affections. Diosmin is rapidly hydrolyzed in the intestine to its aglicone, diosmetin, which is further metabolized to conjugates. In this study, the development and validations of three new methods for the determination of diosmetin, free and after enzymatic deconjugation, and of its potential glucuronide metabolites, diosmetin-3-*O*-glucuronide, diosmetin-7-*O*-glucuronide, and diosmetin-3,7-*O*-glucuronide from human plasma and urine are presented. First, the quantification of diosmetin, free and after deconjugation, was carried out by high-performance liquid chromatography coupled with tandem mass spectrometry, on an Ascentis RP-Amide column (150×2.1 mm, 5 μm), in reversed-phase conditions, after enzymatic digestion. Then glucuronide metabolites from plasma were separated by micro-liquid chromatography coupled with tandem mass spectrometry on a HALO C18 (50×0.3 mm, 2.7 μm, 90 Å) column, after solid-phase extraction. Finally, glucuronides from urine were measured using a Discovery HSF5 (100×2.1 mm, 5 μm) column, after simple dilution with mobile phase. The methods were validated by assessing linearity, accuracy, precision, low limit of quantification, selectivity, extraction recovery, stability, and matrix effects; results in agreement with regulatory (Food and Drug Administration and European Medicines Agency)

guidelines acceptance criteria were obtained in all cases. The methods were applied to a pharmacokinetic study with diosmin (450 mg orally administered tablets). The mean  $C_{\max}$  of diosmetin in plasma was 6,049.3±5,548.6 pg/mL. A very good correlation between measured diosmetin and glucuronide metabolites concentrations was obtained. Diosmetin-3-*O*-glucuronide was identified as a major circulating metabolite of diosmetin in plasma and in urine, and this finding was confirmed by supplementary experiments with differential ion-mobility mass spectrometry.

**Keywords** Diosmetin · Diosmetin glucuronides · Liquid chromatography · Mass spectrometry · Pharmacokinetics · Differential ion-mobility mass spectrometry

## Introduction

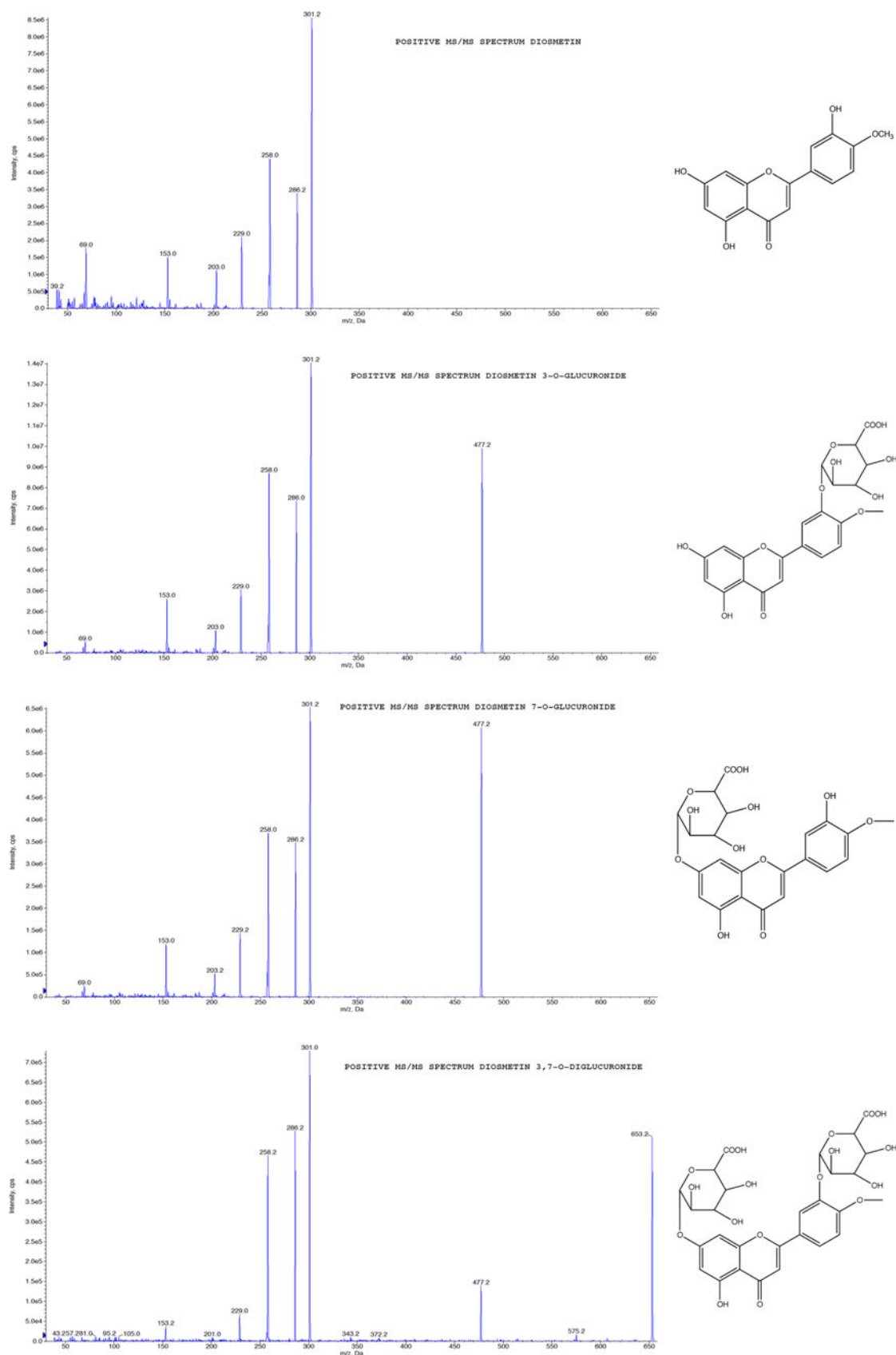
Diosmin is a well-known flavonoid having a broad spectrum of biological activities, including antioxidant, modulator of capillary permeability, and anticarcinogenic [1–3], widely used in medicine. Diosmin formulations, orally administered, are employed for the treatment of chronic venous insufficiency, hemorrhoids, and venous ulcers (especially of the lower limbs) and the prevention of postoperative thromboembolism [4–7]. It is widely used as prescription medicine in Europe mainly for its phlebotropic properties, while in USA is employed as a dietary supplement

Upon ingestion, diosmin is extensively converted to the aglicone diosmetin (chemical formula in Fig. 1) by intestinal bacteria [12] and absorbed as such in the organism.

Diosmetin has been found in plasma as glucuronide conjugates, like almost all flavonoids [8, 9]; several analytical methods, with a digestion step to release diosmetin from the conjugates, have been developed for its quantification [9–13].

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**Fig. 1** Chemical structures and MS/MS spectra of diosmetin, 3-*O*-Gluc, 7-*O*-Gluc, and 3,7-*O*-Digluc

Most authors describe degradation techniques using glucuronidase-sulfatase enzymes (exception Aidong et al. [12] using a chemical degradation); however, Werner et al. published recently a paper raising important concerns on analytical results because of the heavy contamination with diosmetin of enzymes originating from snails [13]. As a matter of fact, extremely different pharmacokinetic (PK) data have been reported in all these studies and the reality is not clear. It is also uncertain what diosmetin metabolites are circulating in body fluids; a single work on identification of diosmetin conjugates in animal samples has been published [14], and another paper mentions, without methodological details, quantitative measurements of diosmetin-3-*O*-glucuronide (3-*O*-Gluc) in human plasma [13] (chemical formulas of the possible glucuronide metabolites can be seen in Fig. 1).

In the present study, the aspects of diosmetin PK and metabolism have been reevaluated in order to clarify the metabolic pathway of this compound and get preliminary PK data. We describe the development and validation of a liquid chromatography–mass spectrometry (LC-MS/MS) method to determine diosmetin in plasma, upon enzymatic degradation with diosmetin-free glucuronidase and sulfatase. Following this, another procedure was developed for the quantitative evaluation of diosmetin glucuronide metabolites in human plasma; a micro-LC-MS/MS system was found suitable for obtaining the required selectivity and sensitivity. The two methods have been applied to a PK study in subjects treated with diosmin (450 mg per os), in order to measure diosmetin concentrations and to define the most relevant type of diosmetin conjugates present in body fluids. Finally, both methods were adapted to may quantify the diosmetin metabolites also in urine with and without an enzymatic step. Diosmetin glucuronides were measured versus calibration curves in biological material spiked with synthetic standards. The monoglucuronides are isobaric and give identical MS/MS fragmentation; since only after the measurements from plasma have been completed, ion mobility mass spectrometry (IMS-MS/MS) became available in our laboratory, supplementary experiments were carried out on urine for confirming the major circulating metabolite. IMS is an excellent tool for the separation of isomers, conformers and isobars as presented in literature [15] and in all manufacturers' application notes.

## Experimental

### Chemicals

Diosmetin (100 %) and paracetamol-3- $\beta$ -D-glucuronide (100 %) were purchased from Sigma (Deisenhofen, Germany), while 3-*O*-Gluc (99.1 %), diosmetin-7-*O*-glucuronide (7-*O*-Gluc;

99.2 %), and diosmetin-3,7-*O*-glucuronide (3,7-*O*-Digluc; 99.4 %) were from Syncom (Groningen, Netherlands).  $^{13}\text{C}_3$ -diosmetin (97.5 %) and  $\text{D}_4$ -paracetamol (99.1%) were acquired from Alsachim (Strasbourg, France) and buprenorphine-3- $\beta$ -D-glucuronide (100  $\mu\text{g/mL}$  in methanol) was from Cerilliant (Round Rock, Texas, USA). All standards used were of analytical grade quality.

Methanol, acetonitrile, 2-propanol, water (all gradient grade for LC), ammonium acetate, dimethylsulfoxide (both analytical grade), formic acid (85 %), and acetic acid (glacial) were purchased from Merck (Darmstadt, Germany).

Glucuronidase (from bovine liver), sulfatase (from Abalone entrails), *p*-nitrocatecholsulfate, 4-nitrocatechol, and D-saccharic acid 1,4-lactone monohydrate were provided by Sigma (Deisenhofen, Germany).

### Standard solutions

Diosmetin, 3-*O*-Gluc, 7-*O*-Gluc, and 3,7-*O*-Digluc stock solutions, at 1 mg/mL, were prepared in dimethylsulfoxide. The stock solutions of internal standards ( $^{13}\text{C}_3$ -diosmetin, buprenorphine-3- $\beta$ -D-glucuronide and paracetamol-3- $\beta$ -D-glucuronide were made in methanol. These solutions were prepared each month and stored at  $-20^\circ\text{C}$ . Working dilutions in solvents or plasma were obtained from the stock solutions when needed.

### Calibration curves and quality control samples preparation

In case of diosmetin determinations in plasma, both without and with enzymatic degradation of the conjugated metabolites, spiked calibration standards (CC) and quality control (QC) samples were prepared with diosmetin at the following concentrations in plasma: 0–50–100–250–750–2,000–6,000–15,000–24,000 pg/mL (CC) and 150–900–9,000–18,000 pg/mL (QC 1, 2, 3, and 4, respectively).

For the direct quantification of diosmetin glucuronide metabolites in plasma, calibration curves were built with 3-*O*-Gluc, 7-*O*-Gluc, and 3,7-*O*-Digluc at the same concentrations, 0–50–100–300–900–2,500–5,000–10,000–15,000 pg/mL and QC at 150–3,000–6,000–12,000 pg/mL (QC 1, 2, 3, and 4, respectively).

For the determination of diosmetin glucuronides in urine, a higher quantification range was used for all three analytes: 0–1–2.667–8–24–72–210–605–1,000 ng/mL for calibration curve concentrations and 3–80–400–800 ng/mL for QC 1, 2, 3, and 4 concentrations. In addition, for the measurement of diosmetin itself, without and with enzymatic digestion, in urine, calibration curves were spiked at 0–10–25–75–200–500–1,000 ng/mL (CC) and QC at 30–375–750 ng/mL (QC 1, 2, and 3, respectively).

## Plasma samples from PK study

A bioavailability study (two periods, two sequences, cross-over, block-randomized, single-dose design), approved by the regulatory authorities and Ethical Committee, was performed in accordance with good clinical practice norms on 12 healthy volunteers treated orally with diosmin (450 mg); subjects received a diet poor in flavonoids during study period (3 days before drug administration and 2 days after). Blood samples (5 mL each) were collected, using heparin as anticoagulant (sampling tubes S-Monovette Hematology 7.5 mL from Sartstedt, Nümbrecht, Germany), before dosing (0.0) then 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0, 16.0, 24.0, and 36.0 h post-dose. Plasma was separated, within 15 min from sampling, by centrifugation (5 min, 2,000 rpm) at 4 °C and aliquots (1–2 mL) were kept frozen at –20 °C until analyzed. Urine samples were collected just before drug administration (basal) and then in the intervals 0–4, 4–8, 8–12, 12–16, and 16–24 h after drug administration.

## Sample preparation procedures

### *Enzymatic degradation of plasma samples*

In the quantitation experiments based on deconjugation, to measure diosmetin in plasma, digestion either with  $\beta$ -D-glucuronidase, or with sulfatase, or with both enzymes together was performed. The sample preparation protocol included in the first step an incubation of a mixture containing 100  $\mu$ L plasma, 25  $\mu$ L glucuronidase, 8 mg/mL (corresponding to an activity of 113.2 U); 25  $\mu$ L sulfatase, 50 units/mL; 10  $\mu$ L paracetamol- $\beta$ -D-glucuronide, 10  $\mu$ g/mL; 40  $\mu$ L *p*-nitrocatechol sulfate, 1 mg/mL (all solutions prepared in sodium acetate 0.2 M, pH 4.5); and 200  $\mu$ L sodium acetate, 0.2 M (pH 4.5), for 12 h at 37 °C, with constant mixing (Memmert). In these experiments, paracetamol- $\beta$ -D-glucuronide was added as an internal standard for the control of the deglucuronidation and *p*-nitrocatechol sulfate for the control of the desulfatation, respectively. The same protocol, but without sulfatase and *p*-nitrocatechol, was applied to evaluate only the diosmetin originating from glucuronide metabolites; in other tests the incubation was repeated also without glucuronidase and paracetamol- $\beta$ -D-glucuronide in order to measure only diosmetin originating from sulfate conjugates (in such cases D-saccharic acid 1,4-lactone monohydrate was added; 0.02 mL at a concentration of 35 mg/mL, during incubation, to inhibit any possible residual glucuronidase activity).

For the calibrators and QC samples, spiked directly with diosmetin, a deconjugation is not needed; therefore, these samples were not incubated with enzymes but just diluted with 0.3 mL sodium acetate 0.2 M, pH 4.5 and further

processed as described in “[Extraction procedure for the determination of diosmetin in plasma.](#)”

### *Extraction procedure for the determination of diosmetin in plasma*

After enzymatic digestion, plasma samples were centrifuged for 1 min at 4,000 rpm. In the case when sulfatase was used, 0.05 mL of the incubated mixture were transferred in a microtiter plate, treated with 0.05 mL NaOH 1 M and the obtained *p*-nitrocatechol was measured at 490 nm to appreciate the desulfatation process efficiency against a standard of *p*-nitrocatechol in water (concentration of 0.32 mMol/L, corresponding to the expected levels of *p*-nitrocatechol achievable in the samples with a complete enzymatic degradation). Then, in all incubated samples 0.02 mL internal standard mixture containing  $^{13}\text{C}_3$ -diosmetin at 200 ng/mL and  $\text{D}_4$ -paracetamol at 2.5  $\mu$ g/mL was added, followed by 0.6 mL acetonitrile. Samples were vortexed for 5 min at 800 rpm, centrifuged 5 min at 4,000 rpm and then 0.8 mL of the clear supernatants were transferred in clean 2-mL 96-well plates and evaporated at 60 °C under an air stream. The dried extracts were reconstituted with 0.15 mL methanol/formic acid 0.1 % in water (1:1, v/v) and injected in the LC-MS/MS system. Paracetamol (resulted from paracetamol- $\beta$ -D-glucuronide degradation) and  $\text{D}_4$ -paracetamol were monitored in the LC-MS/MS method, and the ratio between them was used to evaluate the efficiency of the deglucuronidation process, keeping in mind that equimolar amount of paracetamol and deuterated-paracetamol were added in the reaction and the ionization efficiency of the two analytes.

### *Extraction procedure for the determination of diosmetin-glucuronide metabolites in plasma*

In a separate method, 3-*O*-Gluc, 7-*O*-Gluc, and 3,7-*O*-Digluc were isolated from plasma by a solid-phase extraction (SPE) procedure. Plasma (0.25 mL) was mixed with 0.25 mL formic acid 0.5 % in water and with 30  $\mu$ L internal standard, buprenorphine-3- $\beta$ -D-glucuronide solution 20 ng/mL in methanol, then the samples were loaded under positive pressure onto Isolute C18—100 mg extraction plates (Biotage, Uppsala, Sweden) already conditioned with methanol and formic acid 0.5 % in water. The SPE plates were washed twice with 1 mL formic acid 0.5 % in water and subsequently the analytes were eluted with 0.5 mL methanol/water (80:20, v/v) in clean 2-mL 96-well plates. The eluates were evaporated at 60 °C under a stream of air and reconstituted with 0.15 mL methanol/water (1:1, v/v) containing 0.1 % formic acid.

### *Urine samples processing with enzymatic degradation*

In the quantitation experiments based on deconjugation of diosmetin in urine digestions either with  $\beta$ -D-glucuronidase, with sulfatase, or with both enzymes together were carried out. The sample preparation protocol was identical with the one used for enzymatic degradation of diosmetin conjugates in plasma.

At the end of the incubation period (12 h at 37 °C), to remove proteins, the samples were added with 0.6 mL acetonitrile, mixed for 5 min, and centrifuged for 5 min at 4,000 rpm. Then, 0.2 mL of the clear supernatant were transferred in a clean 2-mL 96-well plate, diluted with other 0.2 mL water containing 0.1 % formic acid and injected in the LC-MS/MS system.

### *Extraction procedure for the determination of diosmetin glucuronide metabolites in urine*

For the urine samples, a dilute-and-shoot approach was used. Urine (0.1 mL) was added with 0.1 mL internal standard solution (paracetamol-3- $\beta$ -D-glucuronide, 1.5  $\mu$ g/mL in water) and 0.8 mL water/methanol (95:5, v/v) containing 2 % acetic acid, mixed for 1 min (800 rpm) and centrifuged for 12 min at 4,500 rpm. Then, 0.2 mL of the clear supernatant were transferred in a clean 2-mL 96-well plate, diluted with other 0.8 mL water/methanol (95:5, v/v) containing 2 % acetic acid and injected in the LC-MS/MS system.

### *Liquid chromatography–mass spectrometry*

Three different instrumental set-ups and chromatographic separations were used in this study. Diosmetin obtained after enzymatic digestion, both from plasma and urine samples, was chromatographed on an amide column (Ascentis RP-Amide 150 $\times$ 2.1 mm, 5  $\mu$ m, Supelco, Bellefonte, USA) eluted with a mobile phase gradient (Method 1). Detailed chromatographic conditions are presented in Table 1. LC-MS/MS analyses were carried out with an Aria LX-2 system developed by Cohesive Technologies (Thermo Fisher Scientific, San Jose, USA), that is integrating through a valve interface module a HTS PAL autosampler and two binary gradient pumps Agilent series 1200 SL (Agilent Technologies, Santa Clara, USA) and allows running two or more samples in parallel. Mass spectrometric detection involved a triple quadrupole instrument model API 4000 (AB Sciex, Toronto, Ontario, Canada) operated in positive electrospray ionization and multiple reaction monitoring (MRM) mode. The transitions 301.11/286.0 and 305.11/286.0 were used to monitor diosmetin and  $^{13}\text{C}_3$ -diosmetin, respectively, while the paracetamol resulted from enzymatic degradation and  $\text{D}_4$ -paracetamol were detected on the transitions 152.02/110.1 and 156.0/114.1, respectively.

Diosmetin glucuronide metabolites extracted from plasma were separated on a microbore octadecyl column (HALO

C18, 50 $\times$ 0.3 mm, 2.7  $\mu$ m, 90 Å), maintained at 50 °C and eluted as described in Table 1 (Method 2). An Ekspert<sup>TM</sup> micro-LC 200 system from Eksigent Technologies (Dublin, California, USA), with HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland), was connected to a triple quadrupole mass spectrometer model API 5000 (AB Sciex) equipped with a 50  $\mu$ m electrospray microprobe. In positive ionization mode, the chromatographic trace 477.2/301.1 was used for both 3-*O*-Gluc and 7-*O*-Gluc while 3,7-*O*-Digluc was monitored on the transition 653.2/301.1 and the internal standard, buprenorphine-3- $\beta$ -D-glucuronide, on the transition 644.3/468.3; the injection volume was 2  $\mu$ L.

Urine analyses of diosmetin glucuronides were also performed using a high-throughput high-performance liquid chromatography (HPLC) system model Aria LX-2 similar to the one described above. Mass spectrometric detection involved a quadrupole-linear ion trap instrument model API 5500 QTrap (AB Sciex) operated in positive electrospray ionization. MRM transition 476.9/286 was used for 3-*O*-Gluc and 7-*O*-Gluc. The trace 653.1/300.8 was used for 3,7-*O*-Digluc and the internal standard of this method, paracetamol-3- $\beta$ -D-glucuronide, was measured on the transition 328.04/151.9. Chromatographic separations were carried out with a pentafluorophenylpropyl column (Discovery HSF5, 100 $\times$ 2.1 mm, 5  $\mu$ m—Supelco) (Method 3, see Table 1); the injection volume was 5  $\mu$ L. Acquisitions were triggered 2 min after sample injection and continued for 4 min; an overlapping of approximately 2 min was obtained with the help of Aria system.

In all aforementioned methods, the eluent from the HPLC column was introduced in the MS interface without splitting; research grade nitrogen was used as curtain and CAD collision gas while auxiliary and nebulizer were supplied with zero-grade air. The samples were kept in the autosampler stack cooled at 10 °C. The optimisation of the mass-dependent parameters for the analytes and their internal standards was carried out by infusing directly in the Turboionspray interface the compounds, diluted at 1  $\mu$ g/mL (or less if needed) in water/methanol (1:1, v/v), with the help of a syringe pump Harvard model “11 PLUS” (Holliston, USA).

Supplementary experiments for the identification of 7-*O*-Gluc and 3-*O*-Gluc were carried out by differential ion-mobility mass spectrometry, using a SelexION interface (AB Sciex) on the API5500 QTrap. Solutions of analytes were infused in the MS source via syringe for parameters optimization or injected via LC system (Method 3).

### *Validation procedures*

All three methods were validated by assessing selectivity, linearity, accuracy, precision, limits of quantification, samples stability in storage and processing conditions, extraction recovery and matrix effects, following European Medicines Agency 2011 [16] and Food and Drug Administration (FDA) 2001 validation



**Table 1** Chromatographic conditions used in the three methods

Method no.	Column type / Temperature	Flow (μl/min)	Time (min)	Mobile phase A (%)	Mobile phase B (%)
Method 1	Ascentis RP-Amide 150x2.1 mm, 5 μm/Room Temperature			Formic acid 0.1 % in water	Methanol
		400	0.0	70	30
		400	0.5	70	30
		400	1.0	20	80
		400	4.0	20	80
		400	4.1	70	30
		400	6.0	70	30
Method 2	HALO C18, 50x0.3 mm, 2.7 μm, 90 Å/50°C			Acetic acid 2 % in water	Acetic acid 2 % in acetonitrile
		35	0.0	98	2
		35	0.3	98	2
		35	3.3	75	25
		35	3.5	20	80
		35	3.8	20	80
		35	4.0	98	2
Method 3	Discovery HSF5, 100x2.1 mm, 5 μm/Room Temperature			Acetic acid 2 % in water	Acetic acid 2 % in methanol
		400	0.0	100	0
		400	1.0	100	0
		400	3.5	20	80
		400	5.0	20	80
		400	5.5	5	95
		400	5.6	5	95
		400	8.1	100	0

guidelines [17]; the FDA/AAPS Crystal City 2007 White Paper recommendations [18] were also considered. The analytical ranges to be validated were chosen based upon the expected plasma or urine concentrations as found in literature [12, 13] and from preliminary experiments. Matrix effects were evaluated comparing the signal of theoretical QC concentrations prepared in mobile phase at 100 % recovery, against the signal of the same concentrations over a blank extract. Matrix factors were calculated for each compound and internal standards (isotope labeled in the case of diosmetin) were used for compensating matrix effects [18, 19].

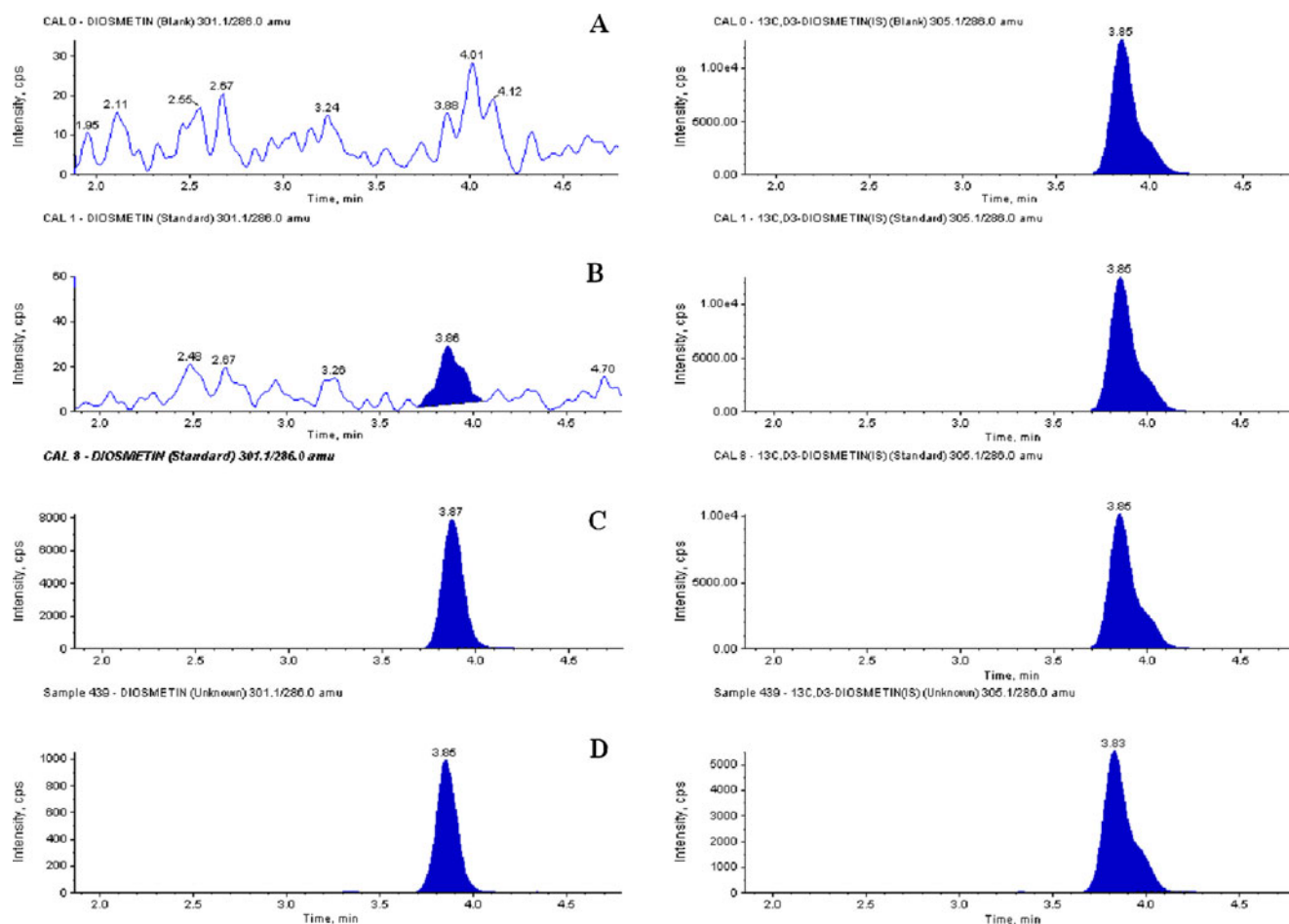
## Results and discussion

### Analytical considerations

Diosmetin (chemical structure and MS/MS spectrum in Fig. 1) is the metabolite of the flavonoid diosmin; compounds of this class are present in most fruits and vegetables, being absorbed in humans mainly as aglicones, with food. Several of the

naturally occurring flavonoid molecules have the same mass of diosmetin (or just a couple of units more like quercetin or hesperetin) and structures differing by the position of the double bonds, giving numerous interferences in its measurement. Moreover, most of these flavonoids are also metabolized as glucuronides.

Our first approach focused on diosmetin determination after enzymatic degradation, and these issues have been reflected in the impossibility to find a totally free blank matrix even if the volunteers included in this study have kept a strict diet. Several stationary phases, from octadecyl (Discovery HSC18) or pentyl (Bio-Wide Pore C5 150×2.1 mm) to amide (Discovery RP-Amide 150×2.1 mm) were tested during development in order to obtain adequate selectivity from background. An anion-exchange column (SAX; 250×4.6 mm) was employed in some experiments for the investigation of diosmetin and metabolites in plasma and urine prior to enzymatic digestion. In the end, taking into account the necessity to use paracetamol as an indicator of deglucuronidation process, the amide column gave the best results and it was preferred for method validation. Chromatograms of extracted



**Fig. 2** Chromatograms recorded on the MRM transitions of diosmetin and its internal standard  $^{13}\text{C}_3\text{D}_3$ -diosmetin after the injection of blank (A), calibrators 1 (50 pg/mL) (B) and 8 (24,000 pg/mL) (C) in plasma, together with a sample from a treated volunteer (D). Column: Discovery

plasma blank or plasma spiked with diosmetin at low (LLOQ) and upper limit of quantification (ULOQ) concentrations are shown in Fig. 2, together with a sample from a treated volunteer, processed by enzymatic degradation and extraction.

Incubation times of 12, 24, and 60 h at 37 °C were tested in order to evaluate the effectiveness of the enzymatic digestion; an incubation of 12 h proved to be sufficient for optimal results. Very important for both glucuronidase and sulfatase digestions was maintaining the pH at 5–5.5, therefore the amounts of plasma, buffer and enzyme used were studied carefully until the final protocols presented in paragraph 2.5.1. In all experiments with glucuronidase or glucuronidase+sulfatase digestions, a ratio paracetamol peak area/ $\text{d}_4$ -paracetamol peak area >0.75 (corresponding to a degradation >85 %) was selected as threshold to accept the individual samples. In case of tests with sulfatase or sulfatase+glucuronidase the absorbance at 490 nm of the digested samples had to be at least 85 % of an equimolar amount of *p*-nitrocatechol, based on the amount of *p*-nitrocatechol sulfate added during incubation.

RP-Amide (150×2.1 mm, 5  $\mu\text{m}$ ); mobile phase gradient of water with 0.1 % formic acid and methanol. Injection volume: 5  $\mu\text{L}$ . Sample digestion and extraction as described in “Sample preparation procedures”

This analytical method (with enzymatic degradation) was validated by assessing selectivity, linearity, accuracy, precision, sensitivity, stability, matrix effects, and extraction recovery; data are summarized in Table 2.

As it can be seen, adequate stability, analytical precision–accuracy, extraction recovery, and matrix effect were obtained. Regarding selectivity, no measurable peaks were recorded at the retention time of the analyte and internal standard. Calibration curves were better calculated using  $1/x$  weighted linear extrapolations and  $r > 0.995$  were always estimated; at LLOQ levels, the accuracy estimated in the six plasma samples from different subjects was 111.8 % with a precision of 6.3 %. To test the influence of the incubation on diosmetin stability in the spiked samples, an experiment with incubated QCs 1 and 4, with and without enzyme, was conducted during validation and the results are presented in Table 2. The QCs incubated in the protocol conditions, with all reagents but no enzyme, were back calculated with good precision and accuracy ( $\pm 15$  %), showing that the analyte was stable. In the case of incubation with enzyme, the back calculated concentration at QC 1 was outside the range

**Table 2** Validation parameters of the method for diosmetin quantification from plasma, after enzymatic degradation

Storage and sample handling stability				
	QC1	QC 2	QC 3	QC 4
Storage conditions (results expressed as % ratio vs. control)				
Plasma at -20 C 2 weeks	111.4 ( <i>n</i> =6)	N/A	N/A	100.6 ( <i>n</i> =6)
Plasma at -20 C 6 months	104.8 ( <i>n</i> =6)	N/A	N/A	101.8 ( <i>n</i> =6)
Plasma freeze-thaw 3 cycles	107.4 ( <i>n</i> =6)	N/A	N/A	99.8 ( <i>n</i> =6)
Plasma 2 h room temperature	103.7 ( <i>n</i> =6)	N/A	N/A	100.9 ( <i>n</i> =6)
Plasma 6 h room temperature	109.6 ( <i>n</i> =6)	N/A	N/A	100.9 ( <i>n</i> =6)
Sample extracts 6 h 10 C	104.7 ( <i>n</i> =6)	N/A	N/A	101.2 ( <i>n</i> =6)
Sample extracts 24 h 10 C	108.0 ( <i>n</i> =6)	N/A	N/A	100.4 ( <i>n</i> =6)
Precision and accuracy				
Within-run mean accuracy (%)	105.8 ( <i>N</i> =6)	98.5 ( <i>N</i> =6)	101.9 ( <i>N</i> =6)	101.8 ( <i>N</i> =6)
Within-run precision (RSD%)	2.8 ( <i>N</i> =6)	1.3 ( <i>N</i> =6)	1.1 ( <i>N</i> =6)	2.9 ( <i>N</i> =6)
Between-run mean accuracy (%)	103.6 ( <i>N</i> =24)	99.3 ( <i>N</i> =24)	101.0 ( <i>N</i> =24)	101.0 ( <i>N</i> =24)
Between-run precision (RSD %)	4.2 ( <i>N</i> =24)	1.9 ( <i>N</i> =24)	1.7 ( <i>N</i> =24)	1.8 ( <i>N</i> =24)
Within-run mean accuracy (%) of incubated QC without enzyme	106.6 ( <i>N</i> =6)	N/A	N/A	99.8 ( <i>N</i> =6)
Within-run precision (RSD %) of incubated QC without enzyme	2.3 ( <i>N</i> =6)	N/A	N/A	1.0 ( <i>N</i> =6)
Within-run mean accuracy (%) of incubated QC with enzyme	139.2 ( <i>N</i> =6)	N/A	N/A	98.8 ( <i>N</i> =6)
Within-run precision (RSD %) of incubated QC with enzyme	2.6 ( <i>N</i> =6)	N/A	N/A	0.8 ( <i>N</i> =6)
Extraction recovery (% ratio vs. unextracted samples)	68.4 ( <i>N</i> =6)	N/A	N/A	73.5 ( <i>N</i> =6)
Matrix effect	0.95 ( <i>N</i> =6)	N/A	N/A	0.94 ( <i>N</i> =6)

(accuracy 139.2 %) as expected, because the endogenous diosmetin glucuronides from plasma were deconjugated, and added to the spiked concentration. These results prove that the approach of not incubating the calibrators and QCs was suitable.

For a comprehensive picture of diosmetin PK, glucuronide metabolites (structures and MS/MS spectra in Fig. 1) in plasma and urine were measured with two separated methods. As a comment to Fig. 1, it is important to mention that no specific fragment ion could be identified to differentiate the two isomers of diosmetin glucuronides, both in positive and negative ionizations; the fragments coming from the loss of glucuronide and then from the fragmentation of diosmetin (in particular by elimination of the methyl group, methanol, or dioxole) being the most relevant. Figure 3 presents the chromatograms recorded using a sub-millimeter column, Halo C18 (0.3×50 mm, 2.7 μm, 90A packing, Eksigent) eluted at 35 μL/min in gradient with a mobile phase containing water+0.5 % formic acid and acetonitrile with 0.5 % formic acid. Chromatograms of extracted plasma blank or plasma spiked with diosmetin glucuronides at LLOQ and ULOQ concentrations are shown, together with a sample from a treated volunteer (same sample presented in Fig. 2).

Four peaks were shown to be distinctly separated in the biological extract within an interval of 0.25 min and diosmetin glucuronides correctly identified; in these conditions, it was possible to obtain a clean blank extract from plasma of patients with special diet. This powerful separation helps in reducing

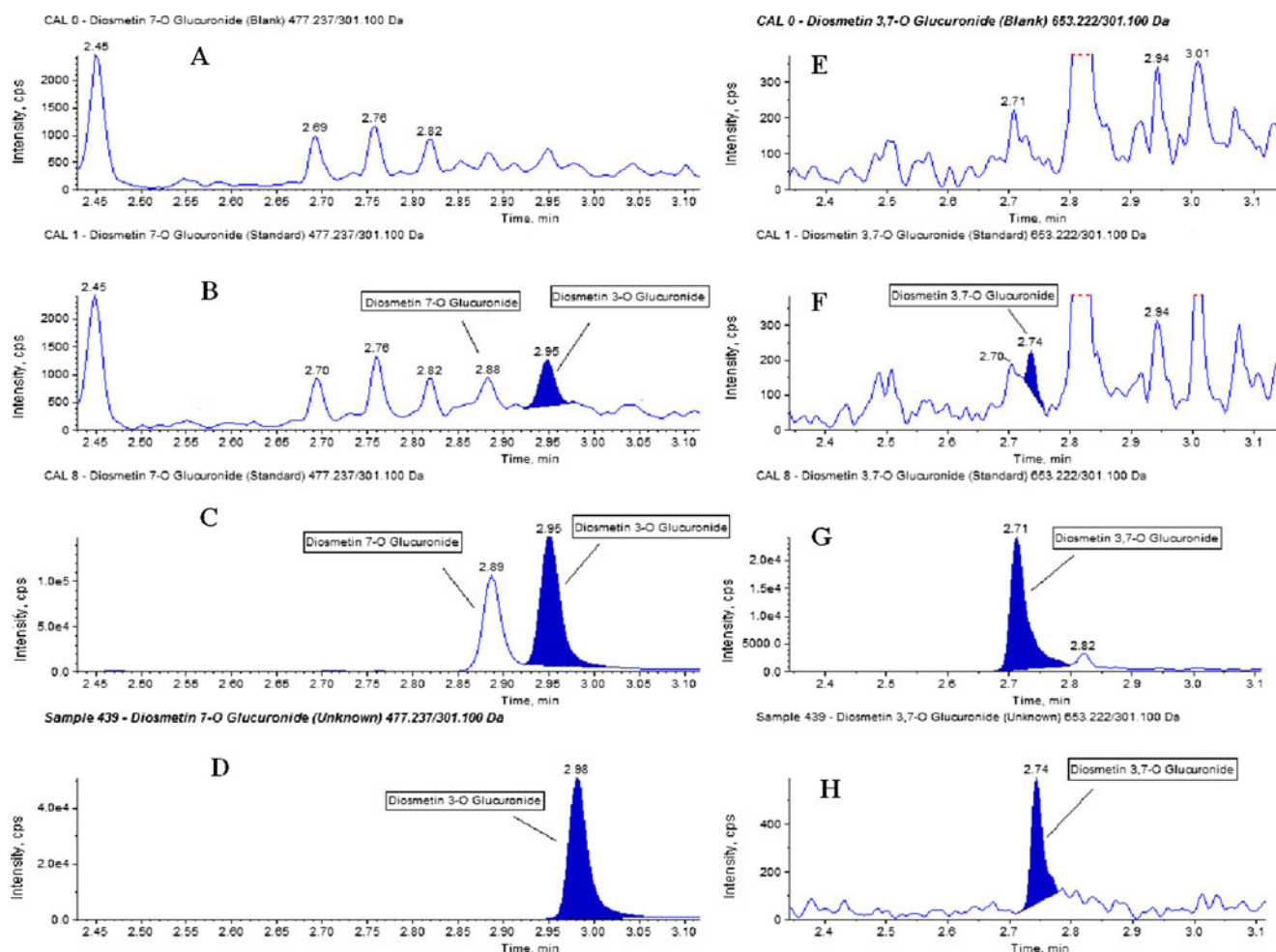
matrix effects and benefits also from the advantage of very low flow rate. Mobile phase consisting of ammonium acetate 10 mM and acetonitrile and negative ionization were tested also with this column but finally the best results in terms of sensitivity and separation were obtained in acidic conditions, positive ions mode. The three glucuronides were isolated from plasma samples by SPE on C18 cartridges in 96-well format as described above in the manuscript. Extraction on anion exchange cartridges (Isolute HAX 100 mg, Biotage, Uppsala, Sweden) was also tested but the high salt content required for elution was detrimental for the chromatography on Halo column.

A complete validation was performed for the quantitative analysis of 3-*O*-Gluc, 7-*O*-Gluc, and 3,7-*O*-Digluc from plasma; a summary of results is presented in Table 3.

Adequate stability, analytical precision / accuracy and matrix effect were obtained. At LLOQ levels, the accuracy estimated in the six plasma samples from different subjects, was 86.0 % with 19.4 % RSD for 3-*O*-Gluc, 90.3 % (8.5 % RSD) for 7-*O*-Gluc, and 111.0 % (5.9 %RSD) for 3,7-*O*-Digluc. PK results measured on real plasma samples will be presented next (“Results on PK samples from a clinical study”).

In the case of urine metabolites concentrations being 10-fold higher (based on enzymatic degradation results), a dilute-and-shoot method was preferred; the microbore column was replaced with a 2-mm pentafluoropropionyl column (Discovery HSF5, 10 cm×2.1 mm, 5 μm) eluted also in acidic conditions as mentioned in “Liquid chromatography-mass





**Fig. 3** Chromatographic traces of 3-*O*-Gluc and 3,7-*O*-Diglu after the injection of blank (a,e), Calibrator 1 (50 pg/mL) (b,f) and Calibrator 8 (15000 pg/mL) (c,g) in plasma, together with a sample from a treated volunteer (d,h). Column: Halo C18 (0.3×50 mm, 2.7 μm, 90a); mobile

phase gradient of water with 0.5 % formic acid and acetonitrile with 0.5 % formic acid. Injection volume: 2 μL. Sample extraction as described in “Sample preparation procedures”

spectrometry.” On this stationary phase, buprenorphine-3-glucuronide was strongly retained and it had to be replaced with paracetamol-3-glucuronide as internal standard. On the transition used to detect diosmetin monoglucuronides in plasma, an interfering peak was observed after injecting urine, thus giving poor chromatographic resolution. In these conditions, a different MRM transition was tested and found suitable for this measurement. Relevant chromatograms are shown in Fig. 4.

For the analysis of urines with enzymatic degradation, the analytical set up used for plasma was not modified. Validations of the analytical methods on urine (with and without enzymatic degradation) were also performed (full validation for glucuronides determination, partial validation for diosmetin quantification upon enzymatic digestion; data presented in Table 4).

Differential ion-mobility mass spectrometry (DMS) was finally used for the confirmation of 3-*O*-Gluc as major metabolite excreted in urine. DMS parameters (separation

voltage, compensation voltage, temperature, modifier, and resolution) were optimized in order to get adequate separation of the isobaric compounds. The use of specific compensation voltages for each of the isomers along with the MRM transition permitted to discriminate the two monoglucuronides and to obtain only one peak on the specific chromatographic trace, instead of two like in the classical MRM, thus increasing the selectivity (Fig. 5).

#### Results on PK samples from a clinical study

##### *Diosmetin determination in plasma after enzymatic degradation*

The mean PK plasma curves of diosmetin obtained from subjects treated orally with diosmin (450 mg) are presented in Fig. 6 together with main PK parameters; the data were obtained analyzing nondigested samples (evaluation of free diosmetin), following glucuronidase digestion (diosmetin

**Table 3** Validation parameters of the method for diosmetin glucuronide metabolites quantification from plasma

	Sample	3- <i>O</i> -Gluc-	7- <i>O</i> -Gluc-	3,7- <i>O</i> -Digluc-
Stability/storage conditions				
Plasma at -20 C 1 month	QC 1 ( <i>n</i> =6)	91.6	104.4	94.5
	QC 4 ( <i>n</i> =6)	97.3	98.7	104.5
Plasma at -20 C 4 months	QC 1 ( <i>n</i> =6)	104.8	100.0	98.3
	QC 4 ( <i>n</i> =6)	91.8	96.9	107.3
Plasma freeze-thaw 3 cycles	QC 1 ( <i>n</i> =6)	98.4	106.3	93.3
	QC 4 ( <i>n</i> =6)	96.3	98.7	106.7
Plasma 1 h room temperature	QC 1 ( <i>n</i> =6)	110.1	108.2	96.5
	QC 4 ( <i>n</i> =6)	104.1	104.4	112.5
Plasma 4 h room temperature	QC 1 ( <i>n</i> =6)	105.0	108.0	95.3
	QC 4 ( <i>n</i> =6)	104.2	106.9	111.8
Sample extracts 8 h room temperature	QC 1 ( <i>n</i> =6)	94.4	98.2	93.5
	QC 4 ( <i>n</i> =6)	90.7	94.0	99.1
Sample extracts 36 h room temperature	QC 1 ( <i>n</i> =6)	86.2	96.2	100.1
	QC 4 ( <i>n</i> =6)	87.4	85.7	95.7
Precision and accuracy				
Within-run mean accuracy (%)	QC 1 ( <i>n</i> =6)	105.8	104.0	101.7
	QC 2 ( <i>n</i> =6)	100.7	104.5	112.6
	QC 3 ( <i>n</i> =6)	99.4	99.4	109.9
	QC 4 ( <i>n</i> =6)	104.0	107.7	108.7
Within-run precision (RSD %)	QC 1 ( <i>n</i> =6)	4.2	3.1	13.3
	QC 2 ( <i>n</i> =6)	3.6	4.4	2.0
	QC 3 ( <i>n</i> =6)	3.9	3.4	4.0
	QC 4 ( <i>n</i> =6)	3.0	5.1	6.5
Between-run mean accuracy (%)	QC 1 ( <i>n</i> =24)	97.1	102.9	99.5
	QC 2 ( <i>n</i> =24)	98.7	103.1	102.2
	QC 3 ( <i>n</i> =24)	100.8	102.4	104.2
	QC 4 ( <i>n</i> =24)	103.9	106.9	104.2
Between-run precision (RSD %)	QC 1 ( <i>n</i> =24)	12.6	6.8	10.5
	QC 2 ( <i>n</i> =24)	6.4	5.1	10.3
	QC 3 ( <i>n</i> =24)	3.8	4.9	8.4
	QC 4 ( <i>n</i> =24)	4.8	4.9	6.8
Extraction recovery (%)	QC 1 ( <i>n</i> =6)	87.4	78.5	47.0
	QC 4 ( <i>n</i> =6)	93.8	81.9	44.5
Matrix effect	QC 1 ( <i>n</i> =6)	0.87	1.36	1.05
	QC 4 ( <i>n</i> =6)	0.89	1.09	1.25

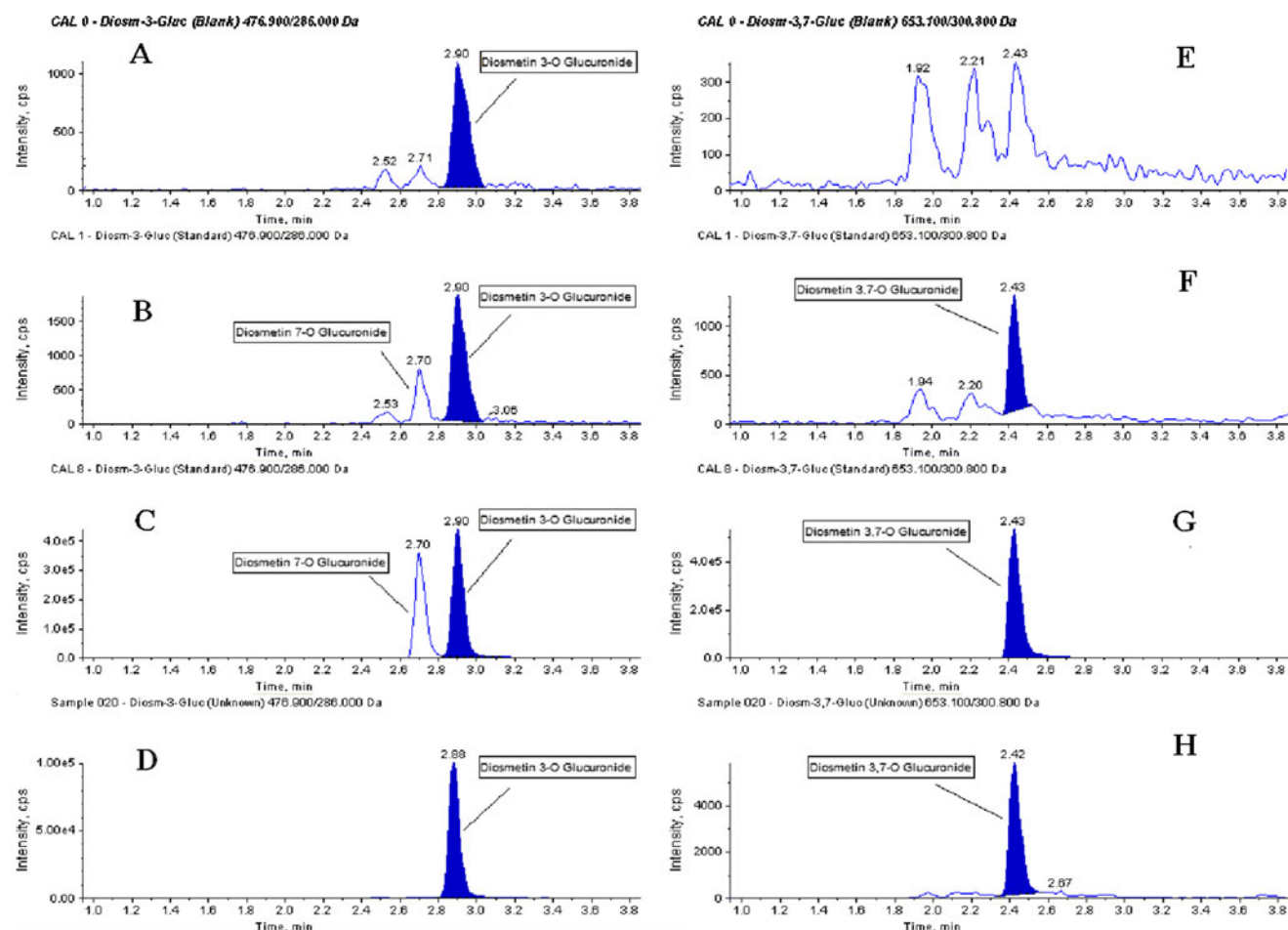
glucuronides levels), sulfatase digestion (diosmetin sulfate levels), and combined sulfatase with glucuronidase digestion (total conjugated diosmetin). As it can be seen, no diosmetin was detected without digestion and as well after sulfatase degradation. Significant levels were measured after glucuronidase and combined sulfatase/glucuronidase degradation; the levels in these two sets of data were very close, almost identical.

It is noteworthy that the concentrations measured are quite close to the ones reported by Wen Aidong et al. and Werner et al. [12, 13]; a few experiments were conducted also on these samples using chemical (acidic) degradation, getting very

similar results. On the other side, the data obtained by Cova et al. [20] or Campanero et al. [10] could not be confirmed. An optimal enzymatic degradation was obtained as confirmed by the addition of paracetamol glucuronide and/or nitrocathecol sulfate.

#### *Diosmetin glucuronides determination in plasma*

The mean PK plasma curves of 3-*O*-Gluc, 7-*O*-Gluc, and 3,7-*O*-Digluc measured in the same samples analyzed first after enzymatic conjugates degradation are reported in Fig. 6, together with main PK parameters.



**Fig. 4** Chromatograms recorded on the MRM transitions of 3-*O*-Gluc and 3,7-*O*-Digluc after the injection of blank (**A**, **E**), calibrators 1 (**B**, **F**) and 8 (**C**, **G**) in urine, together with a sample from a treated volunteer (**D**, **H**). Column: Discovery HSF5 (10 cm×2.1 mm, 5 μm); mobile

The highest levels were observed for 3-*O*-Gluc, while no measurable levels were detected for 7-*O*-Gluc. The diglucuronide of diosmetin was detectable, however, at very low levels, with concentrations at <5 % of those of 3-*O*-Gluc and in the AUC<sub>0-t</sub> calculations the diglucuronide represents only 2.1 % of the total diosmetin glucuronide AUC<sub>0-t</sub>.

A mean PK curve obtained from the experiments performed with glucuronidase digestion (GLUCase diosmetin trace) is also reported in Fig. 6; data can be easily compared with the data of total diosmetin glucuronide (sum of 3-*O*-Gluc and Dio-3-*O*-Gluc); these curves are expressed in pMol/L. As it can be seen, they are very close as further confirmed by the PK data; the AUC<sub>0-t</sub> calculated on diosmetin after glucuronidase digestion is just 3.4 % higher than the one calculated on the sum of the diosmetine glucuronide metabolites (data calculated on molar basis), such difference is within the accepted error of the analytical method.

#### Diosmetin determination in urine after enzymatic degradation

Results obtained by the enzymatic degradation approach on urine samples collected just before and up to 24 hours following diosmin administration are presented in Table 5. Levels before drug administration were practically undetectable and remained quite low up to 4 h from dosing while high concentrations were obtained in the following collections both with glucuronidase and glucuronidase+sulfatase degradation. In case of sulfatase, only modest levels (<5 % of sulfatase+glucorindase concentrations) were observed in the last three urine fractions; no diosmetin concentrations were measured in the undigested samples. The levels after degradation with combined glucuronidase+sulfatase were 10–30 % higher than with glucuronidase only; these results suggest the presence of mixed conjugates, a fact that was not observed in plasma samples.

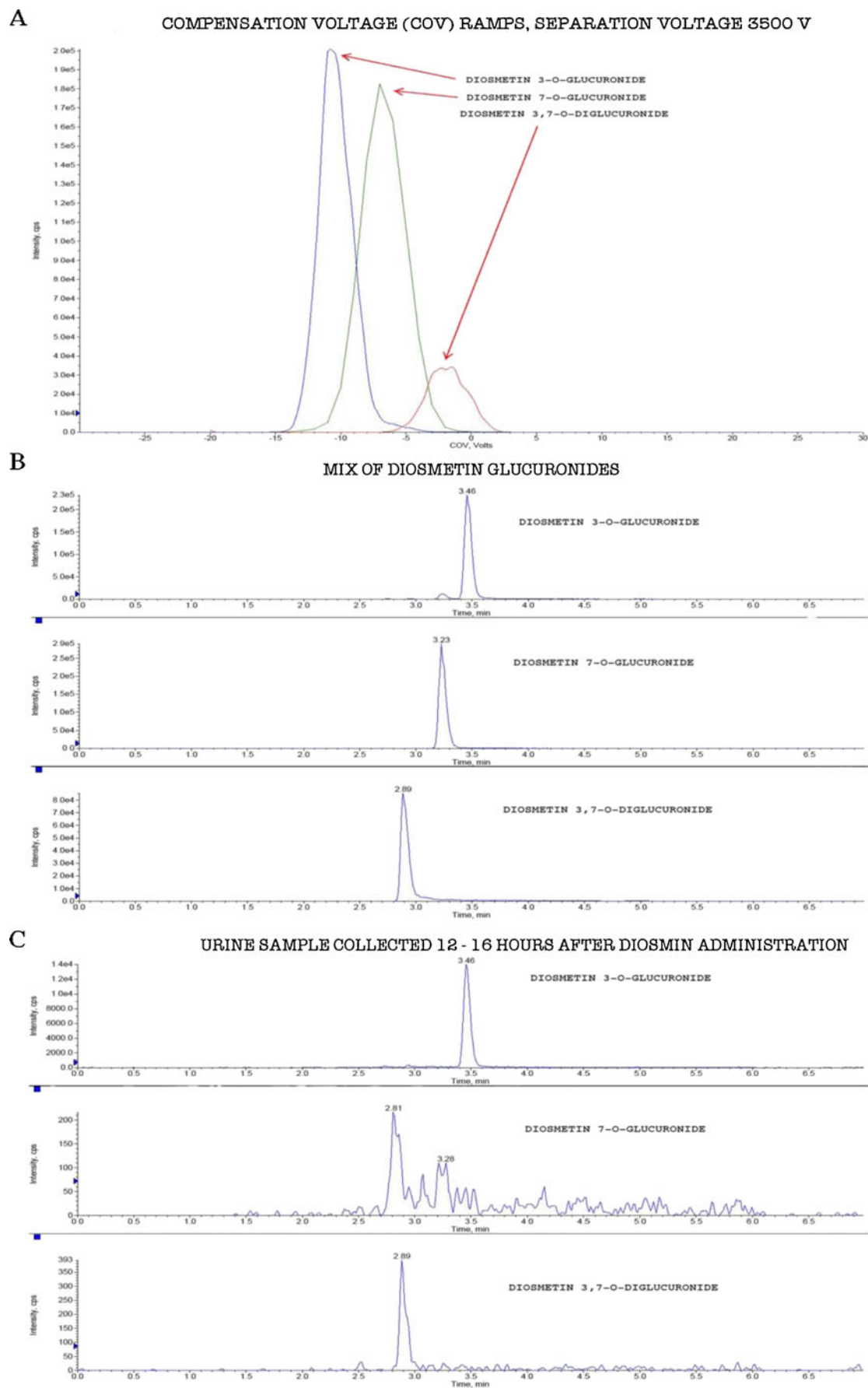
**Table 4** Validation parameters of the method for diosmetin glucuronide metabolites quantification in urine. Partial validation of the method for diosmetin quantification in urine upon enzymatic digestion

	Sample	3- <i>O</i> -Gluc-	7- <i>O</i> -Gluc-	3,7- <i>O</i> -Digluc-
Stability/storage conditions				
Urine at -20 °C 1 month	QC 1 ( <i>n</i> =6)	112.7	106.3	110.4
	QC 4 ( <i>n</i> =6)	91.7	92.0	95.3
Urine at -20 °C 6 months	QC 1 ( <i>n</i> =6)	112.7	100.5	101.1
	QC 4 ( <i>n</i> =6)	87.3	87.9	90.0
Urine freeze-thaw 5 cycles	QC 1 ( <i>n</i> =6)	114.7	108.4	114.6
	QC 4 ( <i>n</i> =6)	94.6	95.4	95.1
Urine 1 h room temperature	QC 1 ( <i>n</i> =6)	111.1	114.6	113.2
	QC 4 ( <i>n</i> =6)	94.4	94.4	94.6
Urine 4 h room temperature	QC 1 ( <i>n</i> =6)	114.0	108.4	95.3
	QC 4 ( <i>n</i> =6)	94.0	95.4	114.1
Sample extracts 8 h 10 °C	QC 1 ( <i>n</i> =6)	108.4	101.1	108.3
	QC 4 ( <i>n</i> =6)	88.5	88.6	94.3
Sample extracts 36 h 10 °C	QC 1 ( <i>n</i> =6)	103.5	105.7	95.9
	QC 4 ( <i>n</i> =6)	86.1	86.9	89.9
Precision and accuracy				
Within-run mean accuracy (%)	QC 1 ( <i>n</i> =6)	103.1	110.4	89.7
	QC 2 ( <i>n</i> =6)	113.1	112.9	114.0
	QC 3 ( <i>n</i> =6)	112.1	111.8	109.9
	QC 4 ( <i>n</i> =6)	94.2	94.0	95.1
Within-run precision (RSD %)	QC 1 ( <i>n</i> =6)	8.6	11.3	11.6
	QC 2 ( <i>n</i> =6)	3.4	3.4	4.0
	QC 3 ( <i>n</i> =6)	0.9	0.8	2.6
	QC 4 ( <i>n</i> =6)	1.0	2.6	2.5
Between-run mean accuracy (%)	QC 1 ( <i>n</i> =24)	110.9	107.9	102.6
	QC 2 ( <i>n</i> =24)	112.3	112.7	112.9
	QC 3 ( <i>n</i> =24)	111.4	112.8	114.6
	QC 4 ( <i>n</i> =24)	92.7	93.1	94.0
Between-run precision (RSD %)	QC 1 ( <i>n</i> =24)	11.2	11.1	13.2
	QC 2 ( <i>n</i> =24)	3.2	3.5	3.9
	QC 3 ( <i>n</i> =24)	2.2	1.7	5.2
	QC 4 ( <i>n</i> =24)	2.4	2.5	2.8
Extraction recovery (%)	QC 1 ( <i>n</i> =6)	77.6	76.9	76.7
	QC 4 ( <i>n</i> =6)	83.3	83.0	82.0
Matrix effect	QC 1 ( <i>n</i> =6)	0.87	1.36	1.05
	QC 4 ( <i>n</i> =6)	0.89	1.09	1.25
Partial validation method with enzymatic digestion		Diosmetin		
Within-run mean accuracy (%)	QC 1 ( <i>n</i> =6)	109.2		
	QC 2 ( <i>n</i> =6)	101.8		
	QC 3 ( <i>n</i> =6)	90.5		
Within-run precision (RSD %)	QC 1 ( <i>n</i> =6)	9.6		
	QC 2 ( <i>n</i> =6)	3.4		
	QC 3 ( <i>n</i> =6)	2.7		

### Diosmetin glucuronides determination in urine

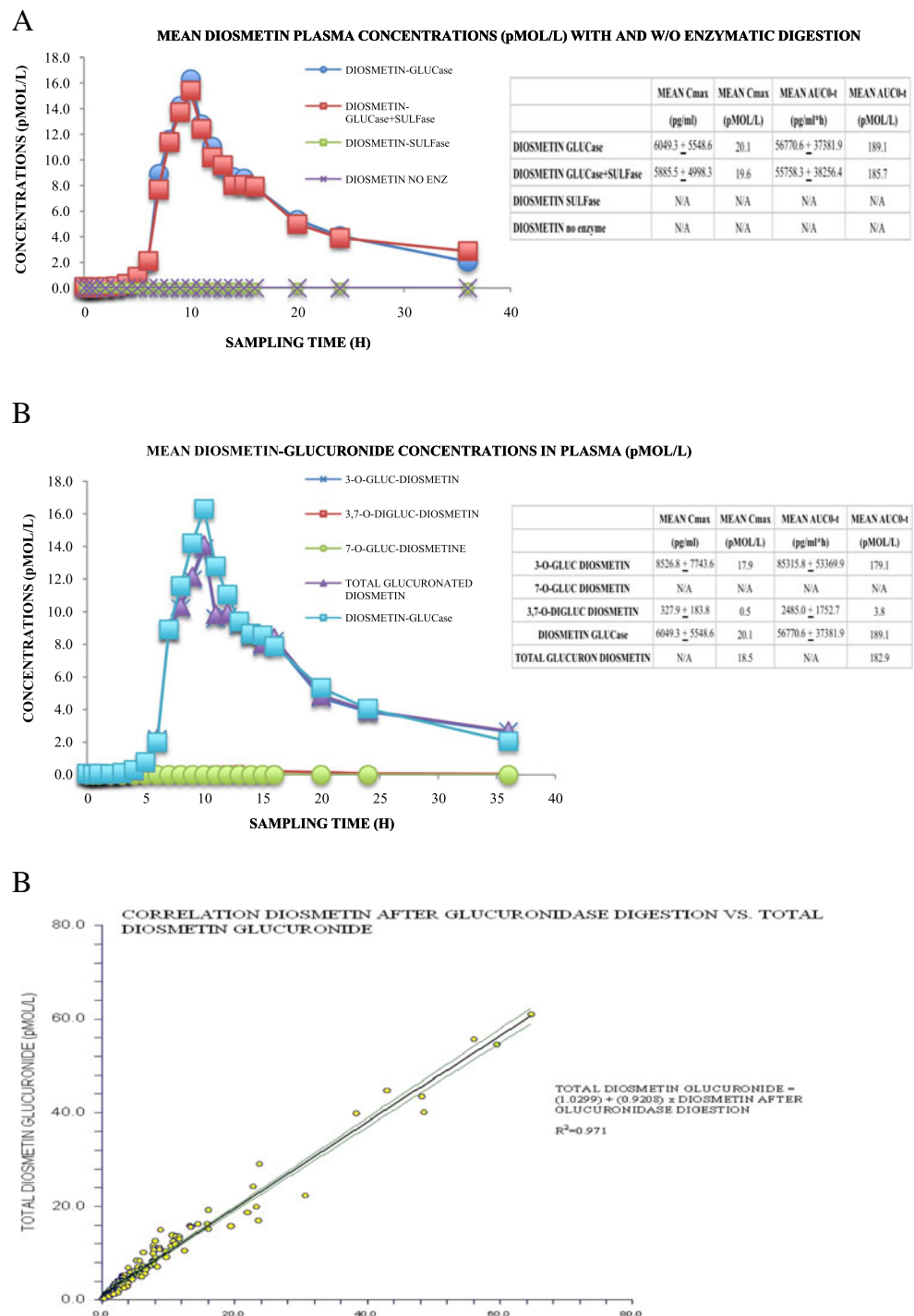
Table 5 shows the measured concentrations of the three glucuronides, the sum of all glucuronide determined, and the diosmetin levels obtained after glucuronidase and sulfatase degradation. Like in plasma, 3-*O*-diosmetin glucuronide was the major metabolite while the 7-*O*-isomer is practically absent; the diglucuronide was measured but the levels were ten times lower compared with the major metabolite.

**Fig. 5** Chromatographic traces of 3-*O*-Gluc (A, D), 7-*O*-Gluc (B, E), and 3,7-*O*-Digluc (C, F) after the injection in the ion mobility-MS interface (SelexION) of a standard mixture (concentration 100 ng/mL) or a urine sample from a treated volunteer. Column: Discovery HSF5 (10 cm×2.1 mm, 5 μm); mobile phase gradient of water with 2 % acetic acid and methanol with 2 % acetic acid. Injection volume, 5 μL. On the top of this figure are presented the compensation voltage ramp data of each analyte, employed to define the optimal DMS separation





**Fig. 6** **A** Mean PK curves of diosmetin measured in plasma after enzymatic degradation and PK parameters obtained on 12 patients orally treated with diosmin 450 mg in a PK study. **B** Mean PK curves of diosmetin glucuronide metabolites measured in plasma and PK parameters obtained on the same 12 patients. **C** Comparison of total diosmetin concentrations obtained with enzymatic degradation or summing the measured glucuronide concentrations (data calculated on molar basis). The evaluation was performed by NCSS 2007 software using a standard linear regression [22] with total diosmetin glucuronide set as the independent variable and diosmetine after glucuronidase digestion as dependent variable. Circles represent individual concentrations measured by LC-MS/MS. Each scatter plot also includes linear regression (*thick line*) and 95 % confidence interval for the regression (dotted lines).  $R^2$  (squared correlation coefficient) represents the proportion of variation in total diosmetin that is accounted by the diosmetin after digestion, and it is presented here as a ratio. Slopes were significantly different from 0 ( $p < 0.005$ ) and consequently the hypothesis that slope=0 was rejected. Good  $R^2$  values were obtained for regression: 0.971. Similar data correlation were obtained performing Deming regression with SAS 9.1 software



The comparison of the glucuronide total levels with those obtained by enzymatic degradation was good but in some cases differences in the range of 30 % were observed; most probable matrix effects in the method without enzymatic degradation were not adequately compensated by the nonlabeled internal standard.

#### Analytical methods correlation

The two analytical approaches (enzymatic digestion vs. sum of the glucuronide concentrations) employed in plasma samples have been statistically compared by NCSS as shown in Fig. 6 where a correlation graph between the obtained diosmetin concentrations is presented. Good  $R^2$  value

**Table 5** Diosmetin mean PK results in urines of subjects treated with diosmin

	Diosmetin no digestion	3- <i>O</i> -Gluc-diosmetin	Diosmetin with glucuronidase	7- <i>O</i> -Gluc-diosmetin	Diosmetin with sulfatase	3,7- <i>O</i> -Digluc-diosmetin	Diosmetin with glucuron. and sulfatase	Total glucuronated diosmetin
Results (pMol/L) with and without enzyme degradation								
Basal urines before treatment	0.0		0.0		0.0		0.0	
Urines collected 0–4 h after treatment	0.0		32.6		0.0		20.5	
Urines collected 4–8 h after treatment	0.0		364.0		0.0		384.9	
Urines collected 8–12 h after treatment	0.0		484.5		3.1		591.0	
Urines collected 12–16 h after treatment	0.0		564.4		6.9		619.3	
Urines collected 16–24 h after treatment	0.0		438.8		19.6		485.5	
Results (pMol/L) of diosmetin glucuronides								
Basal urines before treatment		0.7		0.0		0.1		0.8
Urines collected 0–4 h after treatment		5.7		0.0		0.0		5.7
Urines collected 4–8 h after treatment		207.4		0.0		4.2		211.6
Urines collected 8–12 h after treatment		601.5		1.0		21.2		623.7
Urines collected 12–16 h after treatment		391.5		0.2		12.2		403.9
Urines collected 16–24 h after treatment		358.2		0.5		12.5		371.2

(0.971) was obtained for regression. Since typical linear regression assumes that only one variable can be erroneous, the data were also processed in SAS 9.1, using the Deming regression model in order to validate the results [21]; a correlation of 0.953 was obtained with Deming model.

Based on these results, it can be concluded that diosmetin is essentially present in plasma as 3-*O*-glucuronide with minor levels of 3,7-*O*-diglucuronide while free and sulfates are absent and combined sulfo-glucurono conjugates are questionable.

Also in urine 3-*O*-glucuronide diosmetin is the main metabolite, while the 7-*O*-isomer is absent; interestingly, the levels of diglucuronide were more relevant in urine than in plasma.

## Conclusions

This study reports on the development and validation of three methods for the determination of diosmetin and diosmetin glucuronide metabolites from human plasma and urine. The major circulating metabolite, 3-*O*-Gluc, was identified using synthetic standard and confirmed by differential ion mobility mass spectrometry. To the authors' knowledge, this is the first research presenting complete PK of 3-*O*-Gluc in plasma and urine.

The application of the enzymatic approach put in evidence that glucuronide derivatives of diosmetine are the main metabolites in plasma while sulfates are undetectable as well as the aglicone alone. The experiments in urine confirmed that glucuronides are the prevalent metabolites but also small levels of sulfates were observed and probably mixed conjugate can be found also in very small amounts; the high solubility of the sulfate containing metabolites most probably determine a very fast excretion and so far plasma levels are practically undetectable.

The non-enzymatic method identified 3-*O*-Gluc as the main form circulating in plasma while the 7-*O*-isomer is absent and the di-glucuronide metabolite is at the levels of 2 % of the total glucuronide. It is noteworthy that an optimal correlation was obtained between the analytical method with enzymatic degradation and the method for direct determination of diosmetin glucuronides. Chromatography on sub-millimeter columns and ion mobility mass spectrometry are novel analytical approaches that seem to be very promising for quantitative analysis of complex mixtures and matrices.

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